AN INVESTIGATION OF THE PATTERN OF GROWTH AND MITOSIS OF CELLS OF SARCOMA-180 FROM CROCKER ALBINO MICE GROWN IN VIVO AND IN VITRO

A THESIS

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CHAPTER I

INTRODUCTION

Probably more work is being done on cancer than any other disease. In 1956, Kopac reported that the basic questions asked at the conference on cancer cytology and cytochemistry were: what are cancer cells and what factors divert normal cellular growth into neoplastic growth? Crowdry ('56) indicated in his discussion on malignant properties of cancer cells, that they are cells which characterize malignant tumors irrespective of their origin. Cancer cells make up both carcinomas and sarcomas. A malignant cell (L. malignus wicked) lives at the expense of its host and unless the growth is stopped will cause death. Dulbecco and Vogt ('55) successfully demonstrated that a single virus particle was sufficient to infect a cell. According to Timonen and Therman ('50) malignant cells may be characterized by an imbalance in the timing of the events of the mitotic cycle. Ewing ('40) reported that there may be a focus of embryonic tissue which remained quiescent and did not develop in fetal life but due to some stimulation later in life, tried to assume its normal fetal activity. Tissue culture provides a means by which precise information may be obtained concerning the growth pattern, abnormality in mitoses and effects of inhibitors on the metabolism of cells. The purpose of this investigation was to study the pattern of growth and mitosis of Sarcoma-180 cells grown <u>in vitro</u> and <u>in vivo</u>.

CHAPTER II

REVIEW OF LITERATURE

Jolly ('03) performed experiments which marked the first detailed observation on cell survival and cell division *in vitro*. He successfully maintained leucocytes from the salamander in hanging drops of fowl plasma on a cover slip for approximately a month.

In 1907, Harrison demonstrated that the endings of growing nerve fibers could be observed in the living condition and this provided a means of understanding what took place as the fibers extended. Pieces of neural tissue were taken from frog embryos shortly after the closure of the medullary folds. A fragment of tissue was placed on a cover slip in a drop of fresh lymphatic fluid, taken from the lymph sacs of adult frogs. The lymph quickly formed a clot holding the fragment in a fixed position. The cover slip was inverted over a depression slide and the edges sealed with paraffin. Harrison demonstrated that tissue prepared by this method under aseptic condition could be kept alive and studied from day to day under high magnification.

According to Earle's report in 1943, rat and mouse fibroblasts were successfully grown *in vitro* in a heterologous medium which contained various concentrations of 20-methylcholanthrene. The cells underwent transformation which did not reverse when removed from the carcinogen. Forty days after the first exposure to the carcinogen, the cells were observed to be shorter in length at the terminal processes and the lateral edges were amoeboid in appearance.

Earlier phases of tissue cultivation were concerned mainly with the growth of tissue *in vitro*, but within the last 25 years there has been a resurgence of interest in cancer research. In addition to tissue cultured *in vitro*,

other techniques such as transplantation and the culturing of animal tissue in vivo have also become important in the study of cancer.

Goldie ('48, '49) and Jefferies ('51, '52 and '53) performed experiments in which transplantation of tumor material (Sarcoma-37) in mice produced free tumor cell growth. In these experiments it was demonstrated that free tumor cell growth could be produced by intraperitoneal injections of Sarcoma-37 (implants) into mice. Four days after the initial inoculation, fluid was withdrawn from the peritoneal cavity of the mice and examined. The specimens that showed the highest number of Sarcoma-37 cells were used as donors for the transfer of the cells into other animals. In this report, it was indicated that an ascites tumor is not tissue culture in vivo but a pathological condition of the peritoneal cavity that reveals two phenomena etiologically related, but essentially different. They are: (1) the accumulation in the peritoneal cavity of serous fluid which was due to implantation of tumor cells there and (2) free tumor cell growth in serous fluid due to the transformation of interdependent cells into free tumor cells. According to the investigators (Goldie and Jefferies), ascites tumor did not imply the occurrence of free tumor cell growth, but the growth of free tumor cells was impossible without ascites. It was also indicated that in ascites tumors, the growth of free malignant cells and their spread throughout the body were inseparably associated.

Hesse ('27) demonstrated that Flexner-Jobling Rat Carcinoma can be transmitted by ascitic fluid that developed during the growth of the tumor in the peritoneal cavity. A solid tumor of spontaneous mammary origin (adenocarcinoma) of an inbred strain was minced mechanically and the fresh tumor pulp was injected intraperitoneally into mice belonging to the strain of origin or

a suitable strain. The tumor was maintained by intraperitoneal transfers for at least 10 transfer generations. Fluid was then withdrawn from an ascites tumor of a living animal and transplanted by intraperitoneal injections into other animals. Hesse indicated that this procedure was an easy and convenient means of transplanting tumor material from a donor animal to an experimental animal. He further indicated that fluid could be conveniently withdrawn from the peritoneal cavity of an experimental animal for cytological and chemotherapeutic studies of cancerous cells.

Agate and Agate ('52) demonstrated that mouse Sarcoma-180 could be successfully transplanted into hamsters. Four groups of hamsters of both sexes were adrenalectomized, implanted with 10 mg. pellets of desoxycorticosterone acetate and placed on 0.9% sodium chloride solution substituted for drinking water. The first day, following the operation, a fragment of Sarcoma-180 was implanted subcutaneously in each animal. Three groups that were implanted in the winter, early spring and late fall exhibited continued growth in about 90% of the adrenalectomized hamsters and about 10% of the control animals exhibited continued tumor growth. One group implanted during the summer indicated 50% Sarcoma growth in the experimental animals and 20% in the controls.

The transplantation of human neoplasma into cortisone-treated laboratory animals was demonstrated by Toolan in 1954. A human Sarcoma #1 was obtained from the calf of the leg of a 43 year old male. The soft white mass approximately one cubic centimeter in size was minced and implanted into 7 rats that had been treated with 4 doses of 6 mg. of cortisone. Growth was excellent in all animals and at the end of 15 days enough neoplastic material was available for transfer to 7 additional rats and 20 hamsters.

Stirell ('54) made studies of primary carcinoma in livers of rats using

radioactive materials. The presence of metastatic liver cells were detected in the rats by intravenous injection of radioactive iodinated human albumin into the animal. Twenty four hours after injection, abnormal activity in the cancerous area could be outlined accurately by a scintillascope. Stirell indicated that this method which claimed to be safe and reliable could be used to diagnose primary liver cancer.

About the same time Stirell made his studies with the use of radioactive materials, Sato ('54) made studies on the transplantation of Yoshida Sarcoma in American and Japanese strains of rats. Sato indicated that, during a period of 16 months, intraperitoneal transplantation of Yoshida Sarcoma were maintained in rats for more than 60 generations. From two to 5 days after inoculation, tumors were recognizable and ascites were formed in the peritoneal cavity for from 8 to 14 days. There was evidence in some animals of slight proliferation of cells. However, after several days the proliferation showed evidence of degenerative changes and soon disappeared. Within 8 to 12 days after inoculation, the tumor cells in some American strains indicated spontaneous regression. A similar condition occurred in some of the Japanese strains, and there was a series of similar changes in almost all rats where spontaneous regression of tumor cells occurred.

Mankino and Kano ('51a, '52b) made cytological investigations on ascites tumors in rats, based on the morphological and statistical analyses of chromosomal activity in cancerous cells. Evidence from these studies indicated that stemline cells of tumors were primary contributors to the growth of the tumor. Each tumor studied indicated that tumor cells were characterized by definite chromosomal patterns specific for that kind of tumor, but differed from those of ordinary tissue cells. It was indicated that stemline

cells were capable of regular mitosis in a neoplastic population and were capable of carrying their genetic constitution through transferred generations.

Levan and Houschka ('53) studied the mitotic behavior of histologic spectrum of neoplasma in rats. Evidence indicated that mitosis in each tumor had its own typical pattern with regards to such properties as average amount of heterochromatin in the resting nuclei, chromosome size and state of chromosome contraction. These investigations included studies of the following tumors: carcinoma, sarcoma and lymphomas. Studies of these tumors indicated that chromosomal material observed could be grouped into near diploid and near tetraploid conditions with conspicuous endomitotic activity in the resting nuclei.

CHAPTER III

MATERIALS AND METHODS

The animals used in this investigation were Crocker albino mice secured from Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. The mice were received at the Atlanta University Laboratories on July 19, 1962; the shipment contained 10 female mice of the BALB/cJ strain, and two lots of five donor mice which were inoculated with Sarcoma-180 material on July 17, 1962. All of the mice were 5-weeks-old at the time of arrival. The 10 BALB/cJ female mice were designated as experimental animals and the 5 mice that were inoculated with Sarcoma-180 tumor material were designated as donor animals, from which tumor material used in the transplantations was obtained. Each mouse was placed in a separate container, with identification markings and maintained in a constant temperature room. The animals were fed Purina dog chow and given H_2O *ad libitum*. Their containers were thoroughly cleaned every three days.

The tumors of the donor animals were usually palpable after 5 or 7 days and transplantable after 10 days.

The routine method of tumor transplantation, as outlined by the Roscoe B. Jackson Memorial Laboratory, was used in transplanting tumor material from the donors to the experimental animals. After 5 days, a donor animal was anesthetized with ether and sacrificed, by cutting off the head with a pair of sharp sterile scissors. The mouse was placed in a small battery jar with enough distilled water to cover its body and after a few minutes it was removed from the water and placed in a dissecting pan. The skin in the region of the tumor was swabbed with a piece of cotton dampened with ethyl alcohol.

The tumor was removed with a pair of sterile scissors and forceps and placed in a Petri dish where it was rinsed in Earle's physiological salt solution. A sterile Bard-Parker scalpel was used to cut the tumor into minute pieces, which were put into a trocar (Becton-Dickerson, BD 13 guage needle). All of the instruments used in this experiment were sterilized in a dry sterilizer at 150°C. for two hours to prevent infection of the implant. A different set of instruments were used for each transplantation.

The experimental animal that was to receive the tumor material was anesthetized with ether. The animal was placed in a dissecting pan on its dorsal side and the trocar was inserted subcutaneously in the ventral region of the hip and pushed forward to the axillary region. The minute pieces of tumor material were pushed out by the plunger of the trocar which was withdrawn through a constricted area produced by grasping the skin with the fore finger and thumb just above the point of the trocar. This procedure prevented the implant from being pulled out of place. After the trocar was removed, the area of insertion was swabbed with ethyl alcohol, and the animal was placed in an individual container. Ten days after transplantation, the experimental animal with the palpable tumor was anesthetized with ether and sacrificed by removing the head with a pair of sterile scissors. The mouse was then placed in a dissecting pan and the palpable region of the tumor was swabbed with alcohol and removed. After the tumor was removed from the animal, it was placed in a Petri dish and rinsed in Earle's solution. With a pair of sterile scissors the external area of the tumor was removed and placed in a sectioned Petri dish with a sufficient amount of Earle's solution to keep it moist. The inner most part was placed in a separate section of the Petri dish. A Bard-Parker scalpel (blade No. 10) was used to cut the tissue into small

fragments about one millimeter in diameter. Five cover glasses were placed in a Petri dish top with one drop of fowl plasma and one drop of chick-embryo extract in the center of each as a nutrient medium. One tissue fragment was placed in the medium on each cover glass with a pair of fine iris forceps. The cover glasses were allowed to set for a few minutes in a covered Petri dish, inverted over depression slides and sealed to the slides with paraffin wax. Five mice were sacrificed, one every three days, and from 20 to 25 fragments were explanted during a culture period.

The cultures were placed in an incubator regulated at a constant temperature of 37°C. Cultures were examined daily for growth and cytological changes. At different intervals, cultures were fixed in Bouin's, dehydrated in a series of alcohols and stained with Harris's hematoxylin and eosin for cytological and histological studies.

Slides were prepared from tumors of experimental animals grown in vivo from 10 to 15 days. The mouse was anesthetized with ether and sacrificed by removing the head with a sharp pair of sterile scissors. The skin in the area of the tumor was swabbed with a piece of cotton dampened in alcohol after which the tumor was removed intact with sterile scissors and forceps. The tumor was rinsed with Earle's solution in a Petri dish and transferred to a vial containing Bouin's in which it was fixed for 12 hrs. The tumor was removed from the Bouin's, washed in 35, 50 and 70% alcohol. All slides were prepared and stained by the method outlined in Guyer's Animal Micrology.

CHAPTER IV

EXPERIMENTAL RESULTS

Fragments of a Sarcoma-180 Tumor Grown in vitro

Ninety seven cultures were prepared and observed at various intervals for three 4, 5, and 6 days. Sixty cultures were fixed in Bouin's fluid and stained with Harris's hematoxylin. Thirty one cultures were discarded because of contamination, liquefaction and disintegration.

Culture I.--Twenty four hours after incubation growth was observed at the periphery of 10 explants. Cytoplasmic processes from the explants were observed 48 hrs. after incubation. At the end of three days of incubation, cells which had migrated from the explants individually and in groups were of various sizes and shapes. Many cells were observed to be round, oval and elongated. Some of these cells were binucleated (Fig. 1). The nucleus and/or nuclei of each cell was darkly stained. The individual cells as well as the groups of cells varied in their reaction to the stain used. Some of the cells within the groups of cells showed cytoplasmic vacuolations. Five explants showed no growth at the end of three days and were discarded.

Culture II.--Four days after incubation 19 cultures that were studied in detail showed considerable growth at the edges of the explants. Cytoplasmic processes were well extended from the periphery of each explant. Cells had migrated individually and in groups from the explants. There were variations in the size and shape of individual and groups of cells in that many were club shaped in addition to being round, oval and elongated. Three phases of mitosis were observed namely: metaphase, anaphase and telophase. Some cells had enlarged nuclei and the nuclei within many cells exhibited degeneration. The

cytoplasm of many of the cells was vacuolated (Fig. 2). Six explants were discarded at the end of 4 days, because they exhibited no growth.

Culture III.--Five days after incubation mononucleated and binucleated cells had migrated from the explant almost to the edge of the medium (Fig. 3). The nuclei of some of these cells covered most of the cell bodies and the cytoplasmic processes had extended quite some distance from the explant. The nuclei of the binucleated cells varied in their sizes in that one was larger than the other (Fig. 4). This seemed to indicate an uneven distribution of chromatin material. The nuclear membrane of both mononucleated and binucleated cells were well defined. The cytoplasmic area of many cells differed in their reaction to the stain.

Culture IV.--Six days after incubation the cells observed in 19 cultures were similar to those in Culture III. However, many mononucleated cells exhibited large nuclei and the cytoplasm was granular and highly vacuolated. There were some cells with pyknotic nuclei. There were at least three phases of mitosis observed: metaphase, anaphase and telophase (Fig. 5). Some cells observed in the telophase stage showed one large daughter cell and one small daughter cell. Some cells exhibited nuclear variation in size, form and staining reaction.

Sections of Sarcoma-180 Tumors Grown in vivo

Seventy five slides prepared from tumors grown for 10, 12 and 15 days were studied for cytological details.

A description of sections through a tumor 10 days old.--Sections of the viable portion of the tumor exhibited neoplastic cells which were closely packed in these cords that were separated from each other by a small amount of connective tissue. Cells of the tumor were round or oval with large centrally

located basophilic nuclei. There was a mixture of small cells with pyknotic nuclei and many pleomorphic cells with hyperchromatic nuclei. A few cells exhibited large nuclei surrounded by a clear area and their cellular membranes were not clearly defined (Fig. 6). Traces of muscle tissue were observed between the cells. The central portion of the tumor was necrotic. Cells and their nuclei varied in size, shape and reaction to the stain. Two mitotic figures were frequently observed throughout the sections, metaphase and anaphase.

A description of sections through a tumor 12 days old.--After twelve days of growth cells of various shapes and sizes were observed. Mitotic figures and/or figure were observed throughout the sections (Fig. 7). Relatively large groups of cells partially surrounded by thin strands of connective tissue which contained blood vessels were observed near the periphery of the sections. The cells were nearly all the same size but varied somewhat in shape. The nuclei of some cells were deeply hyperchromatic and fairly uniform in size. The two mitotic figures found most frequently were metaphase and anaphase, and they were usually normal in appearance. The tumor had invaded the muscle tissue remnants of which were found within sections taken from it. A line of demarcation could be observed between necrotic and viable cells.

A description of sections through a tumor 15 days old.--After fifteen days of growth the core of the tumor had disintegrated. There was a demarcation line between the viable and necrotic tissue. The cells varied in shape and size, in that they were usually round, polygonal or sometimes elongated at one or both poles. Mitotic figures usually exhibited the normal forms (Fig. 8). Traces of muscle tissue were more numerous in these sections than in those studied in 10 and 12 day-old tumors. The cells of the necrotic area were darkly stained.

CHAPTER V

DISCUSSION

In this investigation various abnormalities were exhibited in cancerous cells (Sarcoma-180) as compared with normal cells. Hoffman ('50) reported in his studies of cancer cells, that the size of the cell, their nuclei and nucleoli were frequently found enlarged as compared with homologous normal cells. In this study the cells were similar to those described by Hoffman, in that they were very large with large nuclei, however, no nucleoli were observed in any of the cells studied. In Mac Carty's ('29) study of cancerous cells, it was reported that cancer cells were columnar in shape with dense ovoidal or spheroidal nuclei. This correlated with findings made in this study insofar as groups of elongated cells are concerned. These, in addition to being elongated, contained large spheroidal nuclei.

In studies made by Lewis ('37) of cancerous cells grown in vitro using Walker Rat Sarcoma #388, it was reported that they varied in size, shape and detail even in the same culture. According to this investigation, cells when found in groups were elongated, oval, round and club shaped with large nuclei. The cytoplasm of many cells were highly granular.

Cameron ('50) and Cowdry ('58) made a study on the malignant properties of cells. It was reported that malignant cells exhibited a high water content, motility and invasiveness. Grand ('56) reported that cultures of carcinoma of the cervical epithelium showed a greater liquefaction of the clot than appeared in cultures of normal tissue. Liquefaction of many cultures was observed throughout this study. The clot of some cells showed no coagulation and were discarded.

Enterline and Coman ('50) made investigations on the ameboid movement of human and animal neoplastic cells. They reported that frequently, clusters of small cells were found to move in units. In this study individual and groups of cells migrated from the edges of the explants. On an average of from 3 to 9 cells were found in a group. Individual cells frequently migrated to the end of the medium within a period of 6 days. The migration of cells in this study agreed with findings of the above investigators, that indicated invasiveness of cancer cells may be accompanied by ameboid movement of cells that are no longer attached to their mutual adhesiveness.

In studies made by Caspersson and Santesson ('42) on protein metabolism in the cells of epithelial tumors, it was reported that alterations in the staining properties of the nucleus, nucleolus and cytoplasm were due to content type and the distribution of nucleic acids. In this study, cells in both the explants and tumor sections showed variations in their reaction to the stain. The nuclei and portions of the cytoplasm of the cell bodies stained darkly in Harris's hematoxylin, indicating the presence of acids. The central portion of sections stained darker than the outer portion indicating the presence of acids and that necrosis was taking place in this area. Levan ('56) reported, in his studies on chromosomes in cancer cells, that some chromosome numbers showed a greater imbalance than those of normal tissue. Cancer cells exhibited chromosome doublings by endomitosis, endoreduplication or nuclear fusion.

Koller ('47) used Feulgen's technique to study the abnormalities of chromosomes in human tumors. According to him, the abnormalities consisted of the stickiness of chromosomes, irregularity of polyploidy chromosome number and the suppression of the spindle. In studies on chromosomal changes in 174 cases of carcinoma of the female genital tract, Timonen and Therman ('50)

observed lagging chromosomes and the stickiness of chromosomes. In keeping with their findings, it was observed in the present study that a constriction in the nuclei of many cells indicated a condition of lagging chromosomes prior to mitosis. In addition to this finding, it was observed that the nuclei of some cells were very large which indicated a condition of polyploidy. This type of condition was seen in the binucleated cells in that one nucleus was much larger than the other.

In the studies of tumor explants (Sarcoma-180) Patti and Moore ('50) reported that a 7-day-old transplant consisted of a grayish tumor about one centimeter in diameter. A large portion of the central area was hemorrhagic and necrotic; this area was demarcated from the surrounding viable portion by dilated blood bessels. In this study a similar condition was exhibited in sections from 12 and 15-day-old tumors. The central portion in sections from a 12-day-old tumor showed splits and dilated vessels. Sections taken from a 15-day-old tumor showed a degeneration of the central portion. The central two-thirds of sections taken from both tumors stained darker than the surrounding portion indicating that necrosis was taking place.

CHAPTER VI

SUMMARY AND CONCLUSIONS

1. In this investigation Sarcoma-180 was obtained from Crocker albino mice. Minced tumor from donor animals was transplanted into experimental animals where it was allowed to grow for 10 days. After 10 days of growth, a tumor and/or tumors was removed and fragments were taken from it and cultured *in vitro*. Sections taken from 10, 12 and 15-day-old tumors were fixed in Bouin's and stained with Harris's hematoxylin.

2. Cells of the explants were usually found individually or in small groups which showed a variation in size, shape and reaction to the stain. Some nononucleated and binucleated cells migrated quite some distance from the explants. In the prepared slides there was also a variation in size, shape and reaction of cells to stain. The central portion of sections stained darker than the outer portion. This indicated a difference between the viable and necrotic tissue.

3. Cells within some sections taken from a 14-day-old tumor exhibited large spheroidal nuclei that stained darkly and some nuclei covered most of the cell bodies. The nuclear membranes of many cells were not well defined. Traces of muscle tissue were found in 10, 12 and 15-day-old tumors, however, they were more numerous in 15-day-old tumors. All tumors exhibited necrosis, and the core of many showed degeneration.

4. Some cells from both explants and tumor sections showed nuclear constriction as indicated in a lagging of chromosomes and/or nuclear agglutination following mitosis. Many cells showed, during the telophase stage, a variation in the size of the daughter cells, in that one was much larger than

the other. This appeared to indicate some type of mitotic abnormality. The nuclear membranes of the daughter cells were not well defined.

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PLATE I

(Explanation of Figures)*

*All figures are photomicrographs.

2

PLATE II

(Explanation of Figures)*

*All figures are photomicrographs.

3

PLATE III

(Explanation of Figures)*

*All figures are photomicrographs.

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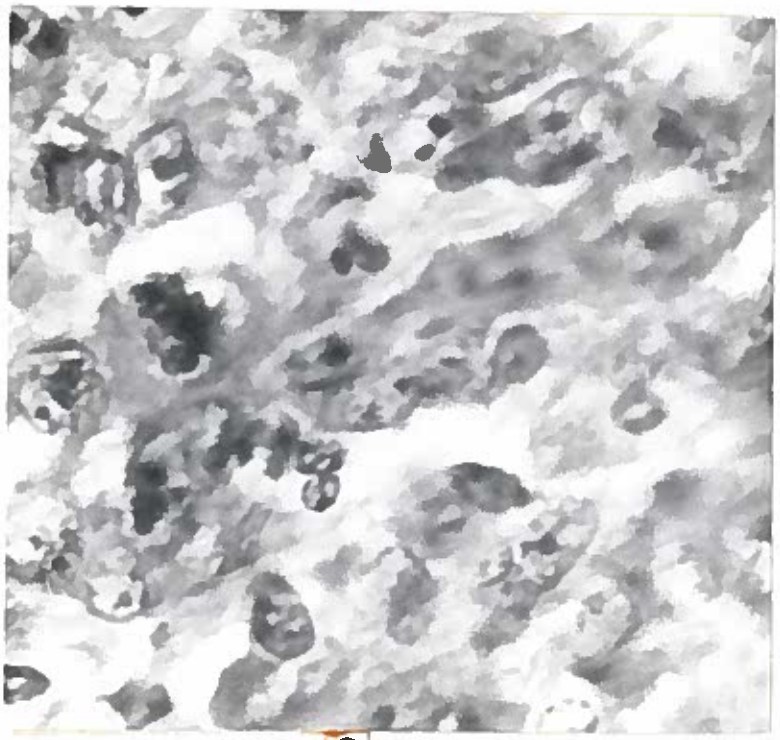

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PLATE IV

(Explanation of Figures)*

*All figures are photomicrographs.